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Epigenetic effects of lung cancer predisposing factors: impact on clinical diagnosis and prognosis

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Abstract

Lung carcinogenesis is a complex process requiring the acquisition of genetic mutations that confer the malignant phenotype as well as epigenetic alterations that may be both manipulated in the course of therapy. Aberrant gene function and transcriptional silencing by CpG island hypermethylation has become a critical component in the initiation and progression of lung cancer. Growing evidence shows that acquired epigenetic abnormalities participate with genetic alterations to cause this dysregulation. Human and animal studies have fostered significant advances in elucidating the role of gene-specific methylation in cancer initiation and progression, the modulation of DNA methylation by carcinogen exposure and the ability of pharmacologic agents to reverse promoter hypermethylation, making it an attractive target to pursue for prevention of lung cancer. This review focuses on how lung cancer predisposing factors participate in epigenetic alterations of lung neoplasia, and discusses the growing implications of these alterations for strategies to control cancer.

Keywords: lung cancer • gene methylation • risk factors • prognosis

Introduction

The term epigenetics describes changes in the expression of genes that are mitotically and/or meiotically heritable, not induced by alterations in nucleotide sequence. The epigenetic regulation of gene transcription is related to DNA methylation and/or histone modifications. These mechanisms affect gene expression during normal cell growth and differentiation but their dysregulation is involved in the pathogenesis of several diseases [1]. Methylation of DNA is a modification that affects the cytosines in the dinucleotide: cytosine–phosphate–guanine (CpG). The covalent addition of a methyl group on the C5 of the Cp transforms it into a methylated cytosine (Cm). This change results in a different global electric charge to the DNA preventing the further binding of the gene transcriptional machinery and subsequent gene silencing. When the change affects tumour-suppressor genes DNA, the cell is ready for malignant transformation [1].

Unscheduled DNA hypermethylation is one of the earliest changes that can be detected along the malignancy process. Since it affects small regions of DNA (about 100 base pairs in size), it is detectable in fresh as in fixed archived tissues samples. Indeed, DNA is a quite robust molecule that is amenable to amplification by polymerase chain reaction (PCR) in order to allow its further detection using fluorescent reporters. Although we do not understand why, how and when only some CpG are changed into Cm in cancer cells, being able to detect them will allow establishing the methylation signature of cancer cells. Moreover, we still do not know all genes where methylation may have consequences with

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regard to potential malignancy. Nevertheless, targeting relevant genes is on the way especially for early lung cancer detection (on induced sputum DNA) of subjects at risk and also on stools DNA to detect colon cancer; early diagnosis in such cases being the key factor for efficient treatments.

In lung cancer, methylation of cytosine residues may affect tumour initiation and progression in several ways, including the silencing of tumour suppressor genes through promoter methylation while providing the targets for adduct formation by polycyclic aromatic hydrocarbons present in combustion products of tobacco smoke. Aberrant DNA methylation has been reported for genes implicated in detoxification of tobacco carcinogens (*CYP1A1*, *GSTP1*), tumour suppression (*p14*, *p15*, *p16*, *p73*, *APC*, *BRCA1*), DNA repair (*hMLH1*, *hMSH2*, *ERCC1*, *MGMT*), differentiation (*GATA*, *RAR*β) and tissues invasion (*CDH1*, *ECAD*, *TIMP1*, *TIMP3*, *LN5*, *DAPK*). These genes have been found methylated in high-risk groups (mainly smokers), premalignant lesions and lung tumours [2–4]. Using microarray analysis in lung cancer cell lines, CpG island methylation was frequently detected for *TIMP4* (64%), *SOX18* (73%), *EGF-like domain 7* (56%), *CD105* (71%), *SEMA2* (55%), *RASSF1A* (71%), *p16* (56%), *SLIT2* (100%) and *TIMP3* (29%) [5]. Methylation was, however, rarely observed in cell lines for *SLIT3* (18%) and *DLC1* (18%) [5]. In primary lung tumours, methylation of *TIMP4* (94%), *SOX18* (100%), EGF-like *domain 7* (100%), *CD105* (69%), *SEMA2* (93%), *DLC1* (61%), *RASSF1A* (44%), *p16* (47%), *SLIT2* (100%) and *TIMP3* (13%) was also detected. Interestingly, inactivation of *RASSF1A* and *p16* correlated well with an extended smoking habit, and exposure to asbestos or squamous cell carcinoma, respectively.

Other epigenetic mechanisms exist that also may affect the function of genes. Initial research has been focused on three pathways, critical for the development of lung tumours: cell-cycle regulation, apoptosis and angiogenesis [6]. Comparative genomic hybridization shows evidence of non-random increases and decreases in chromosomal copy numbers, which was associated with the presence of oncogenes and silence of tumour suppressor genes, respectively [7]. An evolutionary process has been recognized through these molecular changes, leading from normal to malignant phenotype. This process may be linked to inherited or acquired increased mutability or changes in genes transcription activity. One way of exploring this process is to study the epigenetic effects of lung cancer predisposing environmental factors. These include tobacco smoke, radiation, hormones and viral infections (Table 1).

Tobacco smoke

Tobacco is the most studied environmental factor that causes airways epithelium injury, chronic inflammation and field carcinogenesis [8]. Gene expression profiles of non-smokers' adenocarcinoma presented four times more gene changes than tumours of matched smokers, suggesting that tobacco facilitates the emergence of malignant phenotype by acting on normal DNA of the airways cells [9, 10].

Overexpression or increased activity of many oncogenes, including *Myc*, *K-ras*, *EGFR*, *cyclin D1* and *Bcl2*, has been implicated in the pathogenesis of lung cancer as well as abnormal expression or impaired function of tumour suppressor genes, including *p53*, *p16*, *Rb*, *FHIT*, *RASSF1A*, *SEMA3B* and *PTEN* [11]. Tobacco carcinogens may alter epigenetically the expression of these genes. In a study of methylation profile in non-small cell lung cancer (NSCLC), the mean methylation index was found significantly higher in ever smokers than in never smokers, while the mean methylation index of tumours arising in former smokers was significantly lower than the mean of current smokers [12].

GSTP1 protein is highly expressed in lung cancer. In smokers with the *GSTM1* null genotype, the imbalance in the metabolism of tobacco carcinogens may explain the increased susceptibility to lung cancer. In cultures of lung adenocarcinoma cells, induced methylation of *GSTP1* CpG islands reduced the cells viability [13]. The association between functional polymorphisms of *glutathione S-transferase* genes resulting in decreased detoxification of carcinogens or DNA repair and aberrant promoter methylation has been investigated recently. In Chinese female patients, the *GSTP1* G allele in the heterozygous/homozygous state was associated with *RAR*β methylation [14]. This association indicates that in lung cancer there may be a possible link between metabolic pathways of carcinogens and a critical promoter methylation profile.

The p16-INK4a protein inhibits cyclin-dependent kinase 4, a key regulator of progression through the G_1 phase of the cell cycle. The association between methylation of the *p16* promoter region and exposure to tobacco smoke was studied in 185 patients with primary NSCLC where it was significantly associated with smoked pack/years, duration of smoking and negatively with the time since quitting smoking. In patients with stage I lung adenocarcinoma, *p16* methylation was reported to be an independent risk factor, predicting shorter post-operative survival [15].

The tumour suppressor gene *TSLC1/IGSF4* on chromosomal region 11q23 is frequently inactivated by promoter methylation in various cancers, including NSCLC. The promoter was found hypermethylated in 45 (44%) of 103 patients with primary NSCLC and it was significantly associated with the smoking history, cigarettes consumption per day and with a shorter disease-free survival, providing an independent prognostic factor in lung adenocarcinoma patients [16].

The *RAR*β gene encodes one of the primary receptors for retinoic acid, an important signalling molecule in lung growth, differentiation and carcinogenesis. *RAR*β has been shown to be down-regulated by methylation in human lung cancer and it was detected in 54% of preneoplastic hyperplasias induced by treatment with NNK in mice [17]. Bisulfite sequencing of both premalignant and malignant lesions detected dense methylation in the same area observed in cell lines, indicating that this gene is functionally inactivated at the earliest histological stage of adenocarcinoma development [17]. Differential methylation of *RAR*β gene was observed in a study comparing DNA methylation profiles of squamous cell tumours to adjacent normal tissues, in smokers with lung cancer [18]. Furthermore, in patients with NSCLC, low gene expression status of all retinoid receptors (*RAR* and *RXR*)

Table 1 Gene methylation targets of lung cancer predisposing factors

Lung cancer predisposing factor	Methylated gene	Reference
Tobacco smoke	*Cytochrome P4501A1 (CYP1A1)*	[12]
	GSTP1	[13], [14]
	p16-INK4a (*CDKN2A*)	[15]
	TSLC1/IGSF4	[16]
	RARβ	[17], [18]
	O (*6*)*-methylguanine-DNA methyltransferase* (*MGMT*)	[19]
	RASSF1A	[20]
	BLU	[20]
	Fragile histidine triad (*FHIT*)	[21]
Radiation	*p16-INK4a* (*CDKN2A*)	[24]
	Ras effector homolog 1 (*RASSF1A*)	[24]
	GATA5	[24]
	p16-INK4a (*CDKN2A*)	[24]
	MGMT	[24]
Viruses Human papilloma virus (HPV)	*p16-INK4a* (*CDKN2A*)	[25]
Hormones	*Estrogen receptor alpha*	[27]
	Progesterone receptor	[28]

was shown to be a significant independent unfavourable prognostic factor, suggesting a critical dysregulation of the retinoid pathway in lung cancer.

O (6)-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation is a common event in the progression of early stage lung adenocarcinoma. *MGMT* methylation was assessed in smokers with central or peripheral adenocarcinomas, former uranium miners and never-smokers with an overall prevalence for *MGMT* methylation being 51% [19]. The tumour suppressor genes *RASSF1A* and *BLU* are mapped adjacent to one another on chromosome 3p21.3, a region frequently deleted in lung cancer. Hypermethylation of the *RASSF1A* promoter occurred in 47% of the samples of patients with NSCLC (83/178) and of the *BLU* promoter in 43% (68/160) and correlated positively to tobacco consumption, age, gender, histology and starting smoking age under 18 years [20]. A total of 68% of the bronchial lavage samples from the 85 NSCLC patients displayed methylation of at least one of *p16*, *RARβ*, *H-cadherin* and *RASSF1A* genes.

Fragile histidine triad (*FHIT*) gene is a candidate tumour suppressor gene at chromosome 3p14.2 that plays an important albeit controversial role in the pathogenesis of lung cancer. *FHIT* methylation in 125 squamous cell carcinomas (of 254 studied cases) was associated with exposure to tobacco smoke and *p16* methylation [21]. Hypermethylation of *FHIT* in squamous cell carcinomas occurred more frequently in current smokers (45%) than in never-smokers (13%).

Tobacco smoke may also exhibit demethylating effects, inducing the abnormal expression in normally silent genes. *Cytochrome P4501A1* (*CYP1A1*), which is involved in the metabolic activation of polycyclic aromatic hydrocarbon procarcinogens derived from tobacco smoke, is induced in the lung up to 100-fold because of tobacco smoking. Investigation of promoter methylation of CpG sites up to 1.4 kb upstream of *CYP1A1* gene, which contains five potential methylation sites, in lung tissues of smokers, showed complete or partial methylation of *CYP1A1* in 33% of heavy smokers (>15 cigarettes/day), 71% of light smokers (≤15 cigarettes/day or quitted 1–7 days earlier) and in 98% of non-smokers (never and ex-smokers) [12]. There was an inverse correlation between methylation and the number of cigarettes smoked daily. The occurrence of complete methylation was found to increase in 1–7 days after quitting smoking. In active smokers, the partial methylation in the studied region of *CYP1A1* promoter was associated

with a slightly higher pulmonary 7-ethoxyresorufin *O*-deethylase activity (a P450 enzyme that provides a direct method of detection of *CYP1A1*) in the regression models allowing for the daily tobacco consumption and age.

The pro-metastatic oncogene *synuclein-gamma* (*SNCG*) is not expressed in normal lung tissues, but it is highly expressed in lung tumours. Cigarette smoke extract has strong inducing effects on *SNCG* gene expression in lung cancer cells through demethylation of *SNCG* CpG islands. Tobacco exposure may induce the abnormal expression of *SNCG* in lung cancer cells through down-regulation of *DNMT3B* [22]. The *S100A2* gene (a tumour suppressor gene in breast cancer), which encodes a nuclear calcium-binding protein, has demonstrated a diminished expression early during lung carcinogenesis, possibly by hypermethylation of its promoter [23].

Radiation

Lung cancer from radon or plutonium^{239} exposure has been linked to alpha-particles that damage DNA through large deletions and point mutations. Radiation-induced lung adenocarcinoma in workers from MAYAK, a Russian nuclear enterprise [24] has been associated with increased frequency of genes inactivated by promoter hypermethylation. A higher prevalence for methylation of the *p16* gene (*CDKN2A*), the *Ras effector homolog 1* genes (*RASSF1A*) and the tumour suppressor gene *GATA5* was seen in adenocarcinomas from workers compared to tumours from non-worker controls [24]. Stratification of plutonium exposure into tertiles also revealed a dose response for methylation of the *p16* gene.

In addition, methylation studies in sputum cells of uranium-exposed workers has revealed a significant correlation of the *p16* gene methylation rate, the *MGMT* gene methylation rate and the total methylation rate with the increase of the accumulated exposure dosage of the radon daughters compared to controls [24].

Viruses and hormones

Human papilloma virus (HPV) has been found in lung cancer cases with variable frequency. The involvement of HPV infection in lung carcinogenesis of non-smoking females may be mediated through the increase of hypermethylation to cause *p16-INK4a* inactivation [25].

Several lines of evidence suggest that endocrine factors may play a role in the development of lung cancer, but the evidence is limited and inconsistent [26]. It has been documented that *estrogen receptor* (*ER*) transcription silencing due to hypermethylation is linked to the tumour progression of breast, uterine and prostate cancers. *ER* hypermethylation in lung tumours has been associated with the exposure of specific carcinogens in animal studies. The role of hypermethylation-induced *ER* transcription silencing in lung tumour progression and its prognostic value for NSCLC patients are unknown. *ER* hypermethylation was only detected in lung tumours, but not in adjacent normal lung tissues [27]. This suggests that *ER* hypermethylation may be associated with lung tumourigenesis. A high prevalence of *ER* hypermethylation was found in male smoking and non-smoking patients (60 *versus* 61%) as compared to that of female non-smoking patients (34%). Thus, it is possible that anti-estrogens may have different therapeutic values for male and female lung cancer patients. In another study, promoter methylation at the *ER* locus was detected in 4 of 11 tumours from never-smokers and 7 of 35 tumours from smokers [28]. Lung tumours induced by the tobacco-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone also had a low incidence (16.7%) of *ER* methylation, implying that *ER*-specific promoter methylation can be modulated differentially depending on carcinogen exposure. *ERalpha* and *progesterone receptor* are frequently found methylated in mouse lung tumour-derived cell lines. Methylation within these genes was associated with lack of expression that could be restored after treatment with 5-aza-2′-deoxycytidine [28]. Methylation-specific PCR revealed that methylation of these genes occurred at prevalences of 24–69% in primary lung tumours arising spontaneously or induced by exposure to cigarette smoke or NNK.

Implication of gene methylation in clinical diagnosis and prognosis

Recently, epigenetic signatures were studied in an effort to identify new clinically useful markers for lung cancer detection. Although these studies are conducted in a limited number of inhomogeneous subjects and concern different sets of genes, they demonstrate that epigenetic alterations are frequent and easily measurable.

The most recent study by Hsu *et al*. [29] confirmed the effectiveness of screening for lung cancer using hypermethylation as a biomarker. They specifically investigated the methylation status of 6 genes (*BLU*, *CDH13*, *FHIT*, *p16*, *RARβ* and *RASSF1A*), in plasma samples, corresponding tumour tissues and normal lung tissues from a group of 63 patients with lung cancer and in plasma samples from 36 cancer-free individuals. The concordance of methylation in tumour tissues and plasma samples was 86%, 87%, 80%, 75%, 76% and 84% for the *BLU*, *CDH13*, *FHIT*, *p16*, *RARβ* and *RASSF1A* genes, respectively. After several trial tests, the authors established that methylation for $\geqslant$2 of the six markers met the criterion for an elevated risk of cancer. Comparisons yielded a sensitivity of 73%, a specificity of 82% and a concordance of 75% between the methylation patterns in tumour tissues and in corresponding plasma samples. The detection rate was relatively high in cigarette smokers with advanced squamous cell lung cancer.

In sputum from patients with squamous cell lung carcinoma, aberrant methylation of *p16* or *MGMT* was shown to be detectable up to 3 years before diagnosis [30]. Moreover, the prevalence of these markers in sputum of high-risk subjects approximated lifetime risk for lung cancer. In heavy smokers without lung malignancy but with morphometric evidence of sputum cellular atypia, *RARβ* gene was found frequently methylated (27% of the subjects studied), while the methylation frequencies of *CDH13*, *p16* and *RASSF1A* were lower but always

superior from non-smokers controls [31]. In lung cancer patient's sputum, methylation of both *p16* and *RARβ* was shown to be a supplemental diagnostic biomarker for early diagnosis. Paraffin-embedded sputum was also shown suitable for measuring gene methylation in NSCLC patients and smokers with different cytological profile (negative, inflammatory, suspicious or cancer) [32]. *OST-2*, a gene implicated in signal transduction, exhibited the highest levels of promoter methylation in tumours (70% of studied specimens) compared to control tissues. *3-OST-2* and *RASSF1A* showed increased levels of methylation with advanced tumour stage. Sputum DNA analysis from cancer patients further supported these observations. The mismatch repair genes *hMLH1* and *hMSH2* were found methylated in 78% and 26% of studied resected primary NSCLC tumours [33].

The methylation yield of both bronchial lavage and bronchial brushing were inferior in comparison to sputum. Bronchial lavage was also studied for the early detection of tumour-specific methylation. A set of five genes (*p16*, *RASSF1A*, *FHIT*, *RARβ* and *H-cadherin*) were evaluated in resected tumour samples and bronchial lavage of NSCLC patients compared to controls [34]. The majority of tumour samples (68%) showed methylation of at least one of *p16*, *RARβ*, *H-cadherin* and *RASSF1A* genes, while in controls methylation was found mainly for *FHIT* in a minority of tested subjects (28%) and was correlated to their advanced age and smoking history.

Histological subtypes of cancer within a given organ may exhibit distinct methylation patterns and hierarchical clustering of methylation profile of seven genes implicated in lung tumourigenesis, suggesting that methylation patterns differ between NSCLC and SCLC. In patients with surgically resected NSCLC, methylation of *RASSF1A* or *RUNX3* was found to be an independent prognostic factor, as were TNM stage and pleural involvement [35]. In lung tumours samples, the methylation status of three genes was shown to correlate with overall survival. While methylation of *p16* was a predictor of poor survival, methylation of *CDH1* or *TIMP3* genes was a favourable prognostic factor. Adenocarcinoma histotype, older age and smoking history were correlated with a higher methylation index (the ratio between the methylated genes and the number of genes tested). On the contrary, in pleural fluid DNA of NSCLC patients, five tumour suppressor genes (*RASSF1A*, *MGMT*, *p16*, *DAPK* and *RARβ*) failed to show any association with clinical outcome [36]. Sputum was also shown to be superior to serum for predicting gene promoter methylation in stage III NSCLC patients [37]. In the same study, the estimated positive predictive value of a set of four genes that were found most frequently methylated (*p16*, *DAPK*, *PAX5*β and *GATA5*) was 44–72% and the negative predictive value was at least 70%. Sputum data were comparable to those obtained from primary tumour. Similarly, in sputum of late stage NSCLC patients, an aberrant methylation was shown for *ASC/TMS1*, a pro-apoptotic gene. Methylation-dependent transcriptional silencing of *14–3-3sigma*, a major G_2-M checkpoint control gene, was found to be a predictor of longer survival in patients with advanced NSCLC, under chemotherapy.

Modulation of promoter methylation makes it an attractive target to pursue for prevention of lung cancer. Current data regarding lung cancer are limited to animal studies, where short-term treatment with low dose of the demethylating agent 5-aza-2′-deoxyazacytidine (DAC) combined with the HDAC inhibitor sodium phenylbutyrate prevented lung tumour development initiated by 4-methylnitrosamino-1-(3-pyridyl)-1-butanone in the A/J mice [38]. Treatment with DAC alone decreased the incidence of neoplasms by 30% but when DAC was combined with sodium phenylbutyrate, lung tumour development was significantly reduced by >50%. The next step in validating this approach to cancer control will be to determine the efficacy of inhibitors of methylation and histone deacetylation on the progression of premalignant lesions present in the A/J mouse.

Additional demethylating agents that may be used for chemoprevention include small compounds that target the cytosine DNA-methyltransferase genes (DNMTs), which play a critical role in the establishment of the transcriptionally repressive complex by acting as *de novo* methylases. These include zebularine and hydralazine, L-selenomethionine (a nutrient demonstrated to reduce by half the incidence of expected lung cancer, acting in part through inhibition of DNMTs [39]) and the tea polyphenol-epigallocatechin-3-gallate that inhibits DNMTs and reactivates genes silenced by methylation in cancer cell lines [40].

Conclusions – Outlook

Methylation signature analysis already proved to be a potentially powerful diagnostic test for cancer or other life-threatening diseases where altered gene expression is concerned. A remarkably increasing number of scientific reports in this domain emphasize the potential of methylation analysis as a marker of gene expression disorders. Unfortunately, several factors still limit the development of research and applications of methylation analysis and further acceptance as a routine diagnostic and prognostic test. The application of methylation pattern analysis to clinical situations is for the time being limited and additional research will be needed to interpret these findings correctly.

Despite recent progress in epigenetics of lung cancer, a number of important questions remain unanswered. What is the relative part of genomics and epigenomics in the initiation, the regulation and the progression of carcinogenesis? Epigenetic changes are the consequence or the causing factor of gene silencing? Are there distinct tumour-specific and organ-specific methylation signatures depending on the exposition history or the natural course of lung cancer?

Technical questions are also the most important issues including manual standardization of DNA hypermethylation screening and lack of bisulfitation controls. The exploration of CpG islands methylation is insufficient, since hypermethylation can affect equally exons and introns of genes. Hypermethylation of CpG might be progressive, as seen for *FHIT*, were CpG islands hypermethylation

varies in number and location and only sequencing can explore it. Furthermore, MS-PCR is able to detect 2 or 3 CpG each time, being for that cost ineffective for high throughput screening. Progressive elucidation of the aforementioned issues is expected to aiding the use of gene methylation as a pre-screening tool, an early diagnostic test, an assessment of the response to treatment and/or as a scientific basis for the development of new chemoprophylactic or therapeutic agents.

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